Pharmacological Inhibition of the Vacuolar ATPase in Bloodstream-Form Trypanosoma brucei Rescues Genetic Knockdown of Mitochondrial Gene Expression

Pharmacological inhibition of the vacuolar ATPase in bloodstream form *Trypanosoma brucei* rescues genetic knockdown of mitochondrial gene expression

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Running head: Vacuole-mitochondrion crosstalk in *T. brucei*

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[Abstract]

Trypanosomatid parasites cause diseases in humans and livestock. It was reported that partial inhibition of the vacuolar ATPase (V-ATPase) affects dependence of *Trypanosoma brucei* on its mitochondrial genome (kDNA), a target of the anti-trypanosomatid drug isometamidium. Here we report that V-ATPase inhibition with bafilomycin A1 (BafA) provides partial resistance to genetic knockdown of mitochondrial gene expression. BafA does not promote long-term survival after kDNA loss, but in its presence, isometamidium causes less damage to kDNA.

Trypanosomatid parasites cause a range of debilitating or fatal diseases in humans and animals, typically transmitted by insect vectors (1, 2). Important diseases include Chagas disease, caused by *Trypanosoma cruzi*, various forms of leishmaniasis, caused by *Leishmania* spp, Human African Trypanosomiasis (also known as sleeping sickness), caused by *T. brucei* subspecies *T. b. gambiense* and *T. b. rhodesiense*, and the livestock disease nagana, caused by *T. vivax, T. congolense* and, less frequently, *T. b. brucei*.

Trypanosomatids belong to the clade *Kinetoplastea*, flagellated protists characterised by their unusual mitochondrial DNA, called the kinetoplast or kDNA. *T. brucei* kDNA is a concatenated network of dozens of 23-kb maxicircles (the equivalent of mitochondrial DNA in other eukaryotes) and thousands of ~1-kb minicircles that encode guide RNAs (gRNAs). The latter 'guide' post-transcriptional editing of most maxicircle-encoded mRNAs, a process that is essential for generating functional transcripts (3–5). Maintenance and expression of kDNA are essential in both the mammalian bloodstream form (BF) and the insect stage of *T. brucei* (3), and interference with kDNA maintenance is involved in the mode of action of anti-trypanosomatid drugs such as ethidium bromide (EtBr) and

isometamidium chloride (ISM) (6–8). However, BF *T. brucei* appear to require only a single mitochondrial gene product for survival, subunit *a* of the F_O moiety of the F_1F_O-ATP synthase (although translation of the subunit *a* mRNA requires another kDNA-encoded protein, subunit RPS12 of the mitochondrial ribosome). In that stage of the life cycle this complex operates in reverse, as an ATP-driven proton pump, to generate the mitochondrial membrane potential (9–11). Mutations in the nuclearly encoded γ subunit of the ATP synthase, such as L262P, can fully compensate for loss of kDNA in BF *T. brucei* (12) and result in a substantial decrease of ISM sensitivity (7, 13). The mechanism of compensation is not fully understood but appears to involve uncoupling of F_1 from F_O and altered kinetics (11, 12).

Recently, it was reported that perturbation of the vacuolar ATPase (V-ATPase) affects mitochondrial ATPase function and kDNA dependence in trypanosomes. V-ATPase is essential in *T. brucei*, but sub-lethal inhibition of the complex by low-efficiency RNAi or with the V-ATPase inhibitor bafilomycin A1 (BafA) permitted survival for at least 3 days in the presence of normally lethal concentrations of ISM (14). The physiological mechanism of compensation remained obscure. We decided to investigate the effects of sublethal concentrations of BafA on kDNA dependence over longer time scales. All methods were performed as described previously (7, 12) unless specified.

We first investigated whether V-ATPase inhibition affects dependence of BF *T. brucei* on RNA editing. RNA Editing Ligase 1 (REL1) is a key component of the *T. brucei* editosome and its knock-down is lethal (15, 16). Expression of an ATP synthase γ subunit with an L262P mutation fully rescues from this phenotype (12). If partial inhibition of the V-ATPase by BafA renders cells impervious to kDNA loss, then treatment with the drug should also rescue from the growth phenotype observed upon knock down of REL1.

We used a REL1 conditional knock-out cell line (REL1-cKO), where an ectopic copy of the REL1 gene is under control of a tetracycline (Tet)-inducible promoter and both endogenous REL1 alleles have been deleted (15). After removal of Tet from the medium, cKO-REL1 cells exhibited a rapid and severe growth defect, with growth ceasing completely after 96 hours (Fig. 1A, dashed black curve), and no live cells being visible under the microscope at later time points, as observed before (15). The presence of 8 nM or 10 nM BafA alleviated the growth defect, with cells continuing to proliferate 168 hours after Tet removal (Fig. 1A and B, dashed cyan and blue curves and columns, respectively) despite REL1 being below the detection limit in a western blot assay (Fig. 1C and D; all image acquisitions and analyses were performed digitally with Li-Cor Odyssey or C-Digit systems). Lower concentrations of BafA did not alleviate the growth defect caused by REL1 depletion (Fig. 1A, green curves) while higher BafA concentrations caused a severe growth defect even in the presence of Tet (Fig. 1A, pink curves). We note that the range of concentrations in which rescue occurred was narrow and varied slightly between experiments and BafA stocks (data not shown). To investigate if BafA affected knockdown of RNA editing itself, we assessed levels of the F_1F_O ATPase subunit Tb2. The stability of this protein depends on presence of the kDNA-encoded F_O subunit *a*: in the absence of functional kDNA (and thus subunit *a*), the level of Tb2 is substantially reduced (Fig. 1E and F) (17). We found that even in the presence of 8 nM or 10 nM BafA, knockdown of REL1 reduced levels of Tb2 to at least the same extend as loss of kDNA (Fig. 1C). We conclude that the rescue effect provided by BafA appears to reduce dependence on a functional F_1F_O-ATPase, as suggested by Baker et al. (14).

Next, we investigated if treatment with BafA would rescue from cell death caused by kDNA loss, induced either genetically or pharmacologically. To induce kDNA loss genetically, we

repressed expression of TAC102, an essential component of the tripartite attachment complex and thus required for kDNA segregation during cell division (18). As expected, Tet-induced TAC102 knockdown in the published RNAi cell line (18) caused a severe growth defect and kDNA loss (Fig. 2). In contrast to the RNA editing knockdown experiment, however, we did not observe any rescue of either phenotype by incubation with BafA (Fig. 2A and D).

To induce kDNA loss pharmacologically, we treated BF *T. brucei* cells with 0.1 nM ISM. At this concentration, ISM causes kDNA loss but only a very minor growth defect in kDNA-independent cells, as demonstrated by transgenic expression of an F_1 subunit γ allele with the L262P mutation (Fig. 3A, green triangles and lines; see also reference (7)). In the absence of BafA, kDNA-dependent cells showed a severe growth defect: cells complete stopped proliferating after 168 hours (Fig. 3A, black squares and lines), with few, if any, surviving cells visible by microscopic inspection after 240 hours or later. In contrast, in the presence of 10 nM or 15 nM BafA cells continued to proliferate, albeit at much reduced rates (Fig. 3A, blue and orange symbols and lines). The difference in cumulative growth after 313 hours (the endpoint of the experiment) was reproducible and statistically significant (Fig. 3B). At 8 nM BafA the rescue effect was less pronounced (Fig. 3A, cyan triangles and lines, experiment terminated after 192 hours), while 20 nM BafA caused a severe growth defect even in the absence of ISM, as observed before (Fig. 3A and Fig. 1A, pink circles and lines, experiment also terminated after 192 hours). In contrast to what we had found for genetic induction of kDNA loss, we observed that BafA afforded significant protection from kDNA loss caused by ISM. After 96 hours of exposure to ISM, about 70% of control cells had lost their kDNA, as observed by staining with 4',6-diamidino-2-phenylindole (DAPI) and microscopy (Fig. 3C; 0K1N, cells with 1 nucleus but no kinetoplast). In cultures where we added 10 nM or 15 nM BafA to the growth medium

the fraction of 0K1N cells present at 96 hours was reduced to ~50% and ~30%, respectively (Fig. 3C). In cells where a kinetoplast was visible (1K1N cells), quantitation of kDNA by measuring relative fluorescence compared to the nucleus confirmed that exposure to ISM for 96 hours caused a significant reduction in the amount of organellar DNA, presumably an intermediate stage to complete kDNA loss (Fig. 3D). Incubation with 15 nM BafA ISM provided highly significant protection from this effect (Fig. 3D). Taken together, the experiments with kDNA loss induced genetically or by ISM treatment suggest the following: pharmacological inhibition of the V-ATPase with BafA cannot protect *T. brucei* from the lethal effects of complete loss of kDNA. Instead, BafA reduces kDNA damage and loss caused by ISM.

Finally, we induced kDNA loss with an alternative pharmacological regime, exposure to 10 nM EtBr. Like ISM, EtBr is a compound from the phenanthridine class that, at low concentrations, causes kDNA loss but affects growth of kDNA-independent cells only mildly (Fig. 3E, green triangles and lines represent growth of cells expressing a γ subunit with the L262P mutation; see also references (7, 8)). We found that simultaneous incubation with 15 nM BafA resulted in a reproducible rescue from cell death caused by treatment with EtBr (Fig. 3E and F), although the effect was much less pronounced compared to ISM, and very few surviving cells were apparent after 168 hours (the endpoint of this experiment).

These results pose two important questions. First, why did we observe robust rescue by BafA of growth defects caused by knockdown of RNA editing, but not of growth defects caused by inhibition of kDNA maintenance? To our present knowledge both processes serve the same goal in BF *T. brucei*, namely the production of F_1F_O subunit *a* (12). One potential explanation is that partial inhibition of V-ATPase makes the parasites less

dependent on proton pumping by the F_1F_O ATPase, for example by changing the pH balance between cellular compartments, but it does not make the parasites completely independent from it. A similar scenario has been proposed for yeast cells lacking mitochondrial DNA (19). The inducible gene expression system that we used in our study to knock down REL1 is unlikely to achieve complete repression in the absence of inducer (20), presumably resulting in some residual expression of REL1 and, consequently, subunit *a*. In contrast, interference with kDNA maintenance will result in an 'all or nothing' response in affected cells, i.e. cells that lose kDNA will completely lose subunit *a* and F_1F_O-ATPase proton pumping activity. We must note, however, that REL1 in repressed cells was below the detection limit of our western blot analysis, and F_O subunit Tb2 in these cells was reduced to levels comparable to what we observe in cells depleted of kDNA. Testing this hypothesis will require development of more sensitive and highly quantitative methods for detection of intact F_1F_O-ATPase. Second, why was BafA more effective in rescuing the cells from the effects of ISM compared to the related EtBr? Our results provide indirect evidence that V-ATPase inhibition by BafA reduces mitochondrial uptake of ISM. How ISM and EtBr enter the mitochondrion is unknown, although efficient uptake of at least ISM appears to depend on the mitochondrial membrane potential (13). Our results suggest that the mitochondrial uptake mechanisms for ISM and EtBr may not be identical, and that V-ATPase inhibition affects uptake of ISM more than uptake of EtBr. Resolving this question will require development of methods to specifically measure mitochondrial uptake of these compounds.

In summary, our results indicate complex effects of sublethal inhibition of the V-ATPase on *T. brucei* that affect both the degree of dependence on kDNA-encoded products as well as mitochondrial uptake of kDNA-intercalating trypanocides. Our study corroborates an intriguing link between vacuolar and mitochondrial ATPase function that begs further

investigation. Furthermore, we suggest that pharmacological inhibition of V-ATPase function can be a useful research tool in the study of otherwise lethal perturbations of mitochondrial gene expression in BF *T. brucei*, but it should be noted that any experiment will require careful titration of inhibitors.

Acknowledgements

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Figure legends

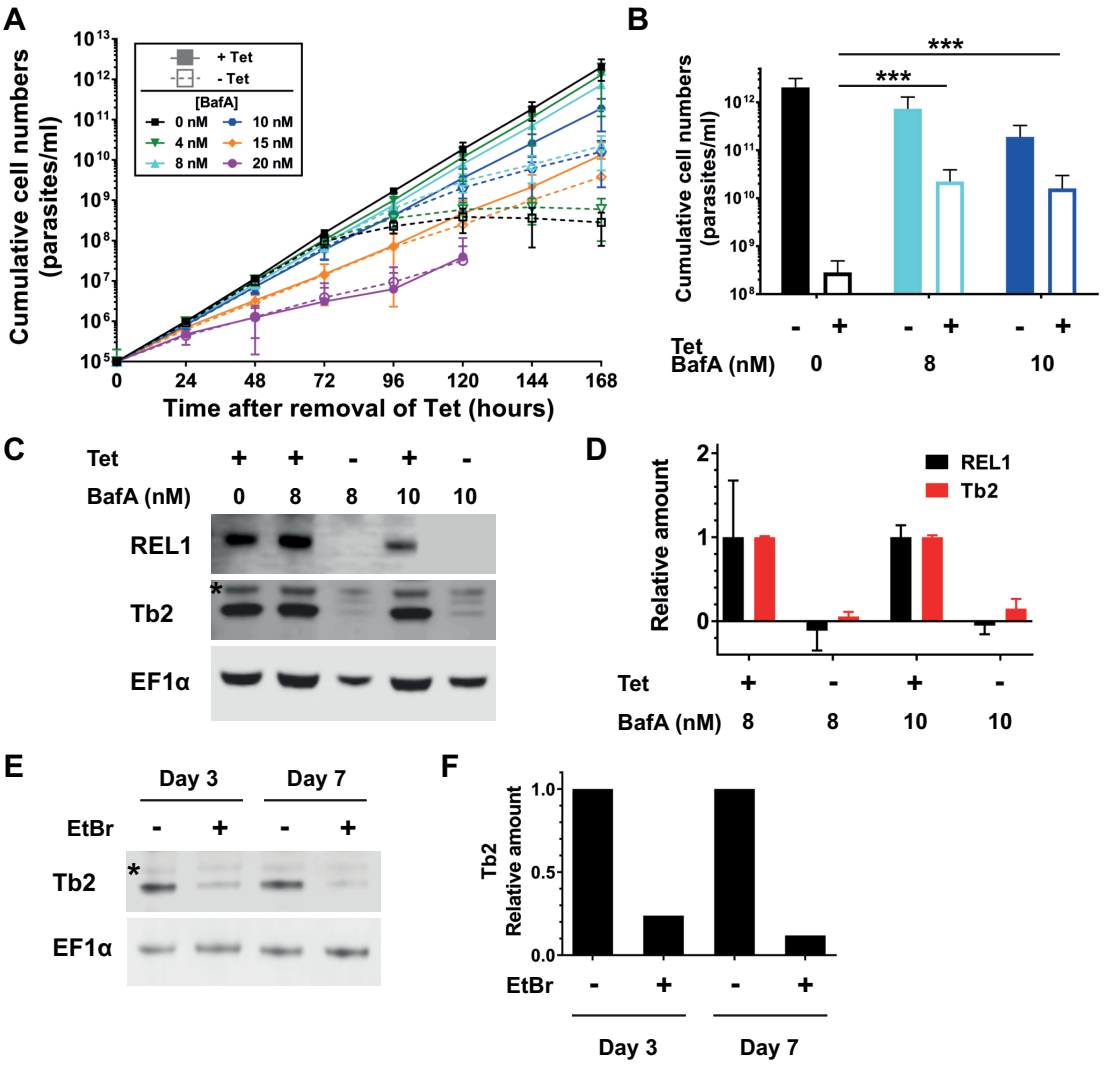
Figure 1. (A) Cumulative growth curves of *T. brucei* REL1-cKO BF cells cultured in the presence (filled symbols, solid lines) and absence (open symbols, dashed lines) of 1 µg/ml tetracycline (Tet; required for expression of REL1) and at varying concentrations of BafA. Each data point is the average of at least six separate growth curves; error bars indicate SD. **(B)** Comparison of cumulative cell numbers (panel A) after 168 hours at 0 nM (n=6), 8 nM (n=8) and 10 nM (n=6) BafA. Statistical significance of differences was assessed with the Wilcoxon rank sum test; $p<0.001$ (***) for -Tet 0 nM BafA vs. -Tet 8 nM BafA and vs. -Tet 10 nM BafA. **(C)** Western blot of samples taken at 0, 8 and 10 nM BafA after 168 hours, probed with a REL1 antibody. The same blot was probed with antibodies for Tb2, to assess levels of intact F_1F_O-ATPase complex (the asterisk indicates a cross-reacting protein), and for EF-1α (Millipore), as a loading control. **(D)**

Quantification of Western blot signals, taking the average of two replicates (one shown in panel C) and indicating relative protein levels under non-induced (-Tet) compared to induced (+Tet) conditions for each BafA concentration (normalized to EF-1α). **(E)** Western blot of samples from *T. brucei* BF cells expressing an ATPase subunit γ allele with the L262P mutation, taken after 3 and 7 days of culturing in the presence of 10 nM EtBr to remove kDNA. Cells grown in the absence of EtBr were used as controls. The blot was probed with antibodies for F_1F_O-ATPase subunit Tb2 (the asterisk indicates a cross-reacting protein, see panel C) and for EF-1α as loading control. **(F)** Relative quantification of the Tb2 western blot signals shown in panel C (normalized to EF-1α).

Figure 2. (A) Cumulative growth curves of *T. brucei* TAC102 RNAi BF cells cultured in the absence (filled symbols, solid lines) and presence (open symbols, dashed lines) of 1 μg/ml Tet and at varying concentrations of BafA. Each data point is the average of 2 growth curves; error bars indicate SD. **(B)** Western blot of samples taken at 0, 8 and 10 nM BafA after 168 hours, probed with antibodies for TAC102 and EF-1α. **(C)** Quantification of Western blot signals, taking the average of two replicates (one shown in panel B) and indicating relative protein levels under RNAi-induced (+Tet) to non-induced (-Tet) conditions for each BafA concentration (normalized to EF-1α). **(D)** Loss of kDNA (0K1N cells) assessed by DAPI staining and microscopy after 168 hours of culturing.

Figure 3. (A) Cumulative growth curves for *T. brucei* BF cells cultured in the absence (filled symbols, solid lines) and presence (open symbols, dashed lines) of 0.1 nM ISM and at varying concentrations of BafA. In parallel, a cell line expressing an ATPase subunit γ allele with the L262P mutation was also grown in the absence of presence of 0.1 nM ISM (green triangles and lines). Each data point is the average of two growth curves, error bars indicate SD. **(B)** Comparison of cumulative cell numbers (panel A) after 313 hours. Statistical significance of differences was assessed with the Student unpaired T-test; $0.01<p<0.05$ (*) for 0 nM BafA +ISM vs. 10 nM BafA +ISM; $p<0.001$ (***) for 0 nM BafA +ISM vs. 15 nM BafA +ISM. **(C)** Loss of kDNA (0K1N cells) assessed by DAPI staining and microscopy after 96 hours of culturing. Statistical

significance of differences was assessed with the Student unpaired T-test; 0.01<p<0.05 (*) for WT +ISM 0 nM BafA vs. WT +ISM 10 nM BafA or vs. WT +ISM 15 nM BafA. **(D)** The relative amount of kDNA in 1K1N cells after 96 hours of culturing was assessed by DAPI staining and quantitation of kinetoplast vs. nucleus fluorescence intensity. Statistical significance of differences was assessed with the Mann-Whitney test; p<0.001 (***) for -ISM 0 nM BafA (n=26) vs. +ISM 0 nM Baf A (n=35); p<0.001 (***) for +ISM 0 nM BafA vs. +ISM 15 nM BafA (n=56). **(E)** Cumulative growth curves of *T. brucei* BF cells cultured in the absence (filled symbols, solid lines) and presence (open symbols, dashed lines) of 10 nM EtBr and at varying concentrations of BafA. In parallel, a cell line expressing an ATPase subunit γ allele with the L262P mutation was also grown in the absence of presence of 10 nM EtBr (green triangles and lines). Each data point is the average from four separate growth curves, error bars indicate SD. **(F)** Comparison of cumulative cell numbers (panel E) after 168 hours. Statistical significance of differences was assessed with the Wilcoxon rank sum test; 0.01<p<0.05 (*) for +EtBr 0 nM BafA vs. +EtBr 15 nM BafA.



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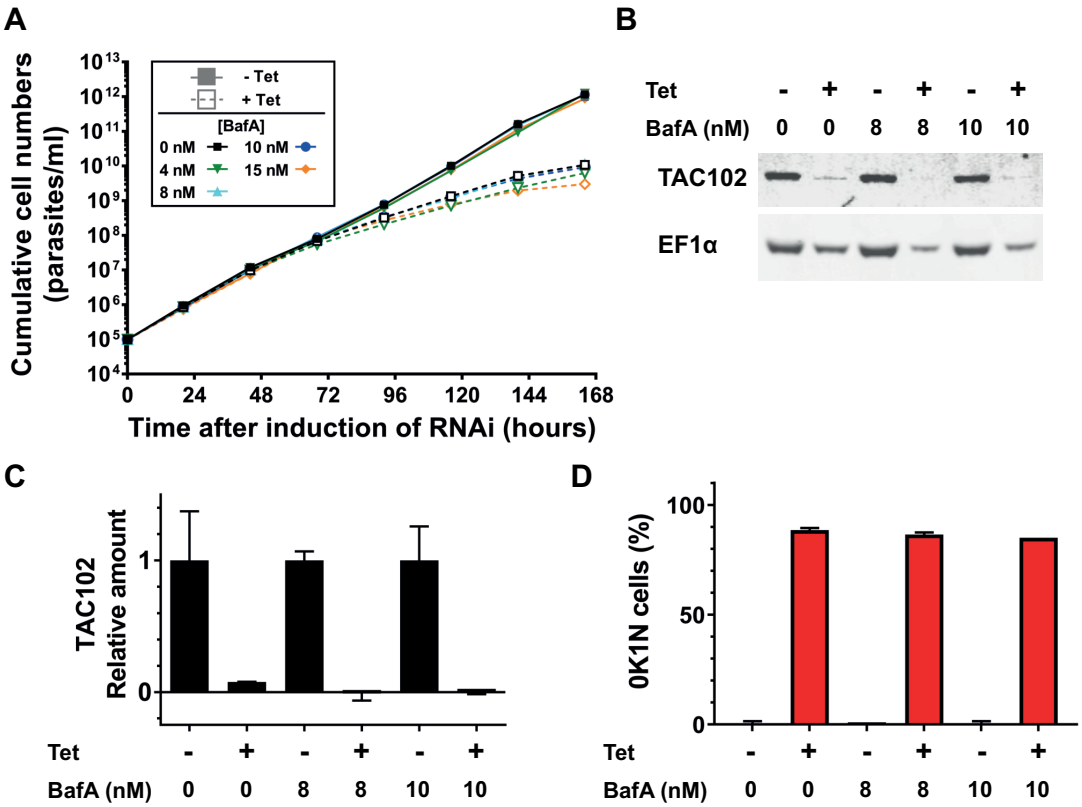

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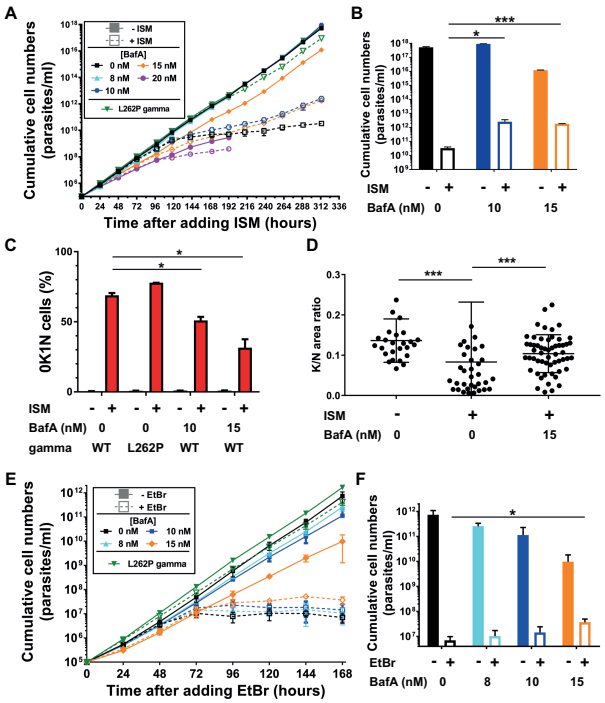

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Figure 3. (continued)
(E) Cumulative growth curves of *T. brucei* BF cells cultured in the absence (filled symbols, solid lines) and presence (open symbols, dashed lines) of 10 nM EtBr and at varying concentrations of BafA. In parallel, a cell line expressing an ATPase subunit γ allele with the L262P mutation was also grown in the absence of presence of 10 nM EtBr (green triangles and lines). Each data point is the average from four separate growth curves, error bars indicate SD. **(F)** Comparison of cumulative cell numbers (panel E) after 168 hours. Statistical significance of differences was assessed with the Wilcoxon rank sum test; 0.01<p<0.05 (*) for +EtBr 0 nM BafA vs. +EtBr 15 nM BafA.

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